

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



Rec'd PCT/PTO

27 JAN 2005



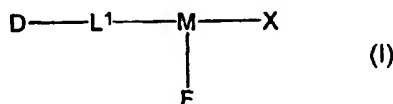
(43) International Publication Date  
5 February 2004 (05.02.2004)

PCT

(10) International Publication Number  
**WO 2004/011946 A1**

- (51) International Patent Classification<sup>7</sup>: **G01N 33/58**, C09B 15/00, C07K 7/06
- (21) International Application Number: **PCT/GB2003/003190**
- (22) International Filing Date: **28 July 2003 (28.07.2003)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:  
0217562.8 30 July 2002 (30.07.2002) GB  
10/241,355 11 September 2002 (11.09.2002) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **SITE-SPECIFIC LABELLING OF PROTEINS USING ACRIDONE AND QUINACRIDONE LIFETIME DYES**



(57) Abstract: Disclosed are compounds of formula: (I) In which D is a fluorescent dye selected from an acridone and a quinacridone dye; F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group; M is a group adapted for attaching to F; X is selected from hydrogen or the group: (II), wherein B is an affinity tag; and L<sup>1</sup> and L<sup>2</sup> each independently comprise a group containing from 1 to 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from -NR<sup>1</sup>-, -O-, -CH=CH-, -CO-NH- and phenylenyl groups, where R<sup>1</sup> is selected from hydrogen and C<sub>1</sub>-C<sub>4</sub> alkyl. The invention also relates to methods that afford direct attachment of the acridone or quinacridone dye to either the N-terminus or C-terminus of a synthetic or recombinant peptide or protein, and their derivatives, in a site-specific manner, coupled with purification of the resultant labelled molecule. Site-specific protein on peptide labelling involves the reaction of a thioester group with a 1,2-aminothiol group as for native chemical ligation.

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Site-specific Labelling of Proteins using Acridone  
and Quinacridone Lifetime Dyes

The present invention relates to reagents and methods for site-specific labelling of proteins with acridone and quinacridone dyes. In particular, the invention relates to new acridone and quinacridone dye derivatives containing thioester activated groups and groups reactive with target molecules containing or derivatised to contain a thioester reactive moiety.

There is an increasing interest in, and demand for, fluorescent labels for use in the labelling and detection of biomolecules. Acridones and quinacridones are highly fluorescent molecules and new acridone and quinacridone dye derivatives having characteristic fluorescence lifetimes have been described for use as labels for target materials. The fluorescence lifetimes of the acridone and quinacridone dyes are generally longer than the lifetimes of other fluorescent labels, as well as naturally occurring fluorescent materials, such as proteins and polynucleotides. The use of fluorescent acridone and quinacridone dyes as fluorescent labels enables easy discrimination from background fluorescence in assays utilising such dyes.

In many applications there is a need to form a permanent link, in the form of a covalent bond, between a fluorescent labelling dye and a target molecule such as a protein. The chemistry of peptide and protein labelling is well documented and a wide range of reagents is now commercially available for the chemical modification of peptides. For a review and examples of protein labelling using fluorescent labelling reagents, see "Non-Radioactive Labelling, a Practical Introduction", Garman, A.J. Academic Press, 1997; "Handbook of Fluorescent Probes and Research Chemicals", Haugland, R.P., Molecular Probes Inc., 1992).

Site-specific incorporation of a fluorescent label into a protein or peptide may be of considerable benefit in certain biochemical and biophysical studies, for example fluorescence resonance energy transfer and protein

structure and function studies. One method for the site-specific attachment of reporter groups into a target polypeptide utilises the native chemical ligation reaction. According to this procedure, an unprotected peptide fragment containing an N-terminal cysteine residue and a second reporter-labelled peptide fragment containing an  $\alpha$ -thioester group are chemoselectively ligated together at physiological pH, irrespective of their primary sequences, to generate an amide bond at the ligation site. For examples, see reviews by Cotton, G.J. and Muir T.W., *Chem. Biol.*, (1999), 6, R247-260; Giriat, I., Muir, T.W. and Perler, F.B., *Genetic Engineering*, (2001), 23, 171-199; Muir, T.W., *Syn. Lett.*, (2001), 6, 733-740.

Tolbert, T.J. and Wong, C-H. (*Angew. Chem. Int. Ed.*, (2002), 41, 2171-2174) describe the preparation of fluorescein and biotin thioester derivatives and the reaction of these with N-terminal cysteine-containing recombinant proteins. Schuler, B. and Pannell, L.K. (*Bioconjugate Chemistry*, 18 July 2002; published on line) reported the preparation of a benzyl thioester of Cy5<sup>TM</sup> and subsequent reaction with a synthetic polypeptide containing an N-terminal cysteine residue.

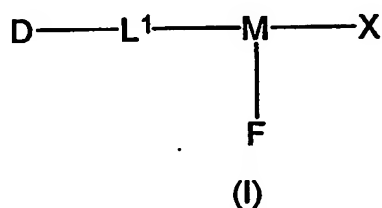
However, there are no reports describing thioester derivatives of fluorescent lifetime reporter molecules such as the acridone and quinacridone dyes, or in which the fluorescence lifetime reporter is additionally linked covalently to an affinity tag. One of the advantages of these reporters is that their characteristic fluorescence lifetime signatures enables target molecules labelled with such dyes to be differentiated from background fluorescence. Additionally, the acridone and quinacridone classes of dyes may be engineered to have a range of fluorescence lifetimes, distinguishable one from the other, thus enabling applications requiring multiplex detection.

The present invention provides reagents and methods that afford direct attachment of a fluorescent acridone or quinacridone dye to either the N-terminus or C-terminus of a synthetic or recombinant peptide or protein, and

their derivatives, in a site-specific manner, coupled with purification of the resultant labelled molecule.

According to one aspect of the present invention, there is provided a compound selected from an acridone and a quinacridone dye containing at least one target bonding group selected from a carboxylic acid thioester group or a group suitable for covalent reaction with a thioester, wherein said compound optionally includes an affinity tag covalently bound thereto.

Suitably, the compound is of formula (I):



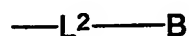
wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

M is a group adapted for attaching to F;

X is selected from hydrogen or the group:

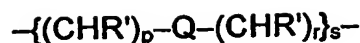


wherein B is an affinity tag; and

L<sup>1</sup> and L<sup>2</sup> each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C<sub>1</sub> – C<sub>4</sub> alkyl.

Suitably, there are 2 to 30 atoms in each of L<sup>1</sup> and L<sup>2</sup>, preferably, 6 to 20 atoms.

Preferably, L<sup>1</sup> and L<sup>2</sup> are independently selected from the group:

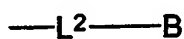


where Q is selected from:  $-\text{CHR}'-$ ,  $-\text{NR}'-$ ,  $-\text{O}-$ ,  $-\text{CH}=\text{CH}-$ ,  $-\text{Ar}-$  and  $-\text{CO}-\text{NH}-$ ; R' is hydrogen or  $\text{C}_1 - \text{C}_4$  alkyl, p is 0 - 5, r is 1 - 5 and s is 1 or 2.

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Particularly preferred Q is selected from:  $-\text{CHR}'-$ ,  $-\text{O}-$  and  $-\text{CO}-\text{NH}-$ , where R' is hereinbefore defined.

10 In a preferred embodiment, group X in the compounds of formula (I) is the group:



wherein B and  $\text{L}^2$  are hereinbefore defined. In this embodiment,  $\text{L}^2$  is optionally a cleavable linker group and may additionally include group P which  
15 may be suitably selected from a chemically-cleavable group, an enzyme-cleavable group, or a photochemically-cleavable group. Suitable chemically cleavable groups include carbamate esters and carboxylate esters, which are both cleaved under basic conditions. Suitable enzyme cleavable groups may be selected from groups such as ester, amide and phospho-diester groups.  
20 Such groups are substrates for, and are hydrolysed by hydrolases, such as proteases, esterases and phospho-diesterases. Suitable photocleavable groups P for use in the compound of formula (I) may contain the 4,5-dialkoxy-2-nitrobenzyl alcohol linker (Holmes, C.P., and Jones, D.G., J.Org.Chem., (1995), 60, 2318-2319) or phenacyl linkers (Wang, S., J.Org.Chem., (1976),  
25 41, 3258-3261). These groups undergo efficient photoreaction upon 300nm illumination, resulting in the rapid cleavage of the dye molecule or dye-labelled protein from the affinity tag.

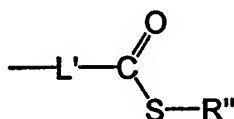
30 Suitably, the group M may be any suitable functional group adapted for attaching the target bonding group F. Preferably, M is selected from:



wherein R' is hereinbefore defined.

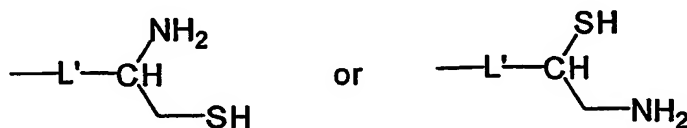
Suitable affinity tags may be selected from biotin, desthiobiotin and metal chelating ligands such as his-tag and iminodiacetic acid, nitrilotriacetic acid and the like. Preferred affinity tags may be selected from biotin and desthiobiotin.

In one embodiment of the present invention, the target bonding group F is a carboxylic acid thioester of formula:



wherein L' is a bond or is a group containing from 1 – 30 linked atoms selected from carbon atoms and optionally one or more groups selected from –NH–, –O– and –CO–NH–; and R'' is C<sub>1</sub> – C<sub>4</sub> alkyl, C<sub>6</sub> – C<sub>10</sub> aryl, or C<sub>7</sub> – C<sub>15</sub> aralkyl, which may be optionally substituted with sulphonate; or is the group –(CH<sub>2</sub>)<sub>2</sub>–CONH<sub>2</sub>. In the case where L' is a bond, the target bonding group F is attached directly to group M.

In an alternative embodiment, the target bonding group F is a 1,2-aminothiol group of formula:

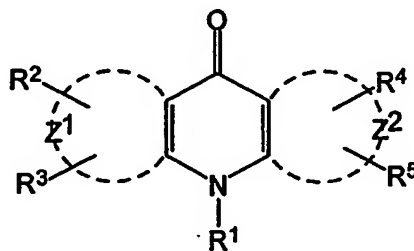


wherein L' is hereinbefore defined.

Thus, the present invention provides fluorescent labelling reagents comprising an acridone dye or a quinacridone dye, modified by incorporating

a target bonding group, and optionally an affinity tag into the molecule. The target bonding group may be selected from a carboxylic acid thioester group or a 1,2-aminothiol group, wherein the thioester group is selectively reactive with a 1,2-aminothiol group on a target molecule, suitably a protein or peptide, or a derivative thereof. In the alternative, the acridone or quinacridone dye may contain a 1,2-aminothiol group for reaction with a thioester group on the target. The incorporation of a reactive thioester or, alternatively, a 1,2-aminothiol functionality into the chemical structure of the reporter groups enables the target molecule to be directly labelled in a convenient one step process. According to the methods of the invention, labelling of peptides and proteins is site-specific, irrespective of the composition of the primary sequence. By generating the target primary sequence with either an N-terminal cysteine or a thioester functionality, site-specific labelling can be achieved directly, by incubating the target with the appropriate derivative of the acridone or quinacridone dye, the  $\alpha$ -thioester and 1,2-aminothiol derivatives respectively. The inclusion of an affinity tag in the labelling reagent allows subsequent purification of the fluorescent dye-labelled protein or peptide.

In one embodiment according to the first aspect, the compound is an acridone dye having the formula (II):

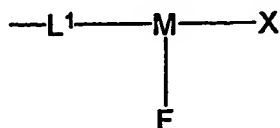


wherein:

groups  $R^2$  and  $R^3$  are attached to the  $Z^1$  ring structure and groups  $R^4$  and  $R^5$  are attached to the  $Z^2$  ring structure;  
 $Z^1$  and  $Z^2$  independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or

six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;  
at least one of groups  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  is a group W having the formula:

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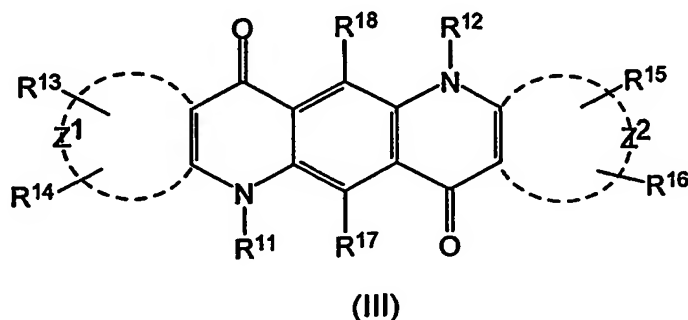


where F, M, X and  $L^1$  are hereinbefore defined;

- 10 when any of said groups  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  is not said group W, said remaining groups  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  are independently selected from hydrogen, halogen, amide, cyano, mono- or di- $C_1 - C_4$  alkyl-substituted amino, carbonyl, carboxyl,  $C_1 - C_6$  alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl,  $C_1 - C_{20}$  alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group  $-(CH_2)_n-Y$  and,
- 15 when group  $R^1$  is not said group W, it is selected from hydrogen,  $C_1 - C_{20}$  alkyl, aralkyl and the group  $-(CH_2)_n-Y$ ; and Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;
- 20 provided that at least one of groups  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  is a water solubilising group.

In a second embodiment according to the first aspect, the compound is a quinacridone dye having the formula (III):

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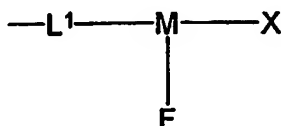
30

wherein:

groups  $R^{13}$  and  $R^{14}$  are attached to the  $Z^1$  ring structure and groups  $R^{15}$  and  $R^{16}$  are attached to the  $Z^2$  ring structure;

$Z^1$  and  $Z^2$  independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$  and  $R^{18}$  is a group T having the formula:



where F, M, X and  $L^1$  are hereinbefore defined;

when any of said groups  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$  and  $R^{18}$  is not said group T,

said remaining groups  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$  and  $R^{18}$  are independently selected from hydrogen, halogen, amide, cyano, mono- or di- $C_1 - C_4$  alkyl-substituted amino, carbonyl, carboxyl,  $C_1 - C_6$  alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl,  $C_1 - C_{20}$  alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group  $-(CH_2)_n-Y$ ; and,

when either of groups  $R^{11}$  and  $R^{12}$  is not said group T, it is selected from hydrogen,  $C_1 - C_{20}$  alkyl, aralkyl and the group  $-(CH_2)_n-Y$ ;

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

provided that at least one of groups  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$  and  $R^{18}$  is a water solubilising group.

Suitably, in the compounds according to formula (II) and (III),  $Z^1$  and  $Z^2$  may be selected independently from the group consisting of phenyl, pyridinyl, naphthyl, anthranlyl, indenyl, fluorenyl, quinolinyl, indolyl, benzothiophenyl, benzofuranyl and benzimidazolyl moieties. Additional one, or two fused ring systems will be readily apparent to the skilled person. Preferably,  $Z^1$  and  $Z^2$  are selected from the group consisting of phenyl, pyridinyl, naphthyl, quinolinyl

and indolyl moieties. Particularly preferred  $Z^1$  and  $Z^2$  are phenyl and naphthyl moieties.

Suitably, at least one of the R groups of the dyes of formula (II) and (III) is a water solubilising group for conferring a hydrophilic characteristic to the compound. Solubilising groups, for example, sulphonate, sulphonic acid and quaternary ammonium, may be attached directly to the aromatic ring structures  $Z^1$  and/or  $Z^2$  of the compound of formula (I) and (II). Alternatively, solubilising groups may be attached by means of a  $C_1$  to  $C_6$  alkyl linker chain to said aromatic ring structures and may be selected from the group  $-(CH_2)_n-Y$  where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6. Alternative solubilising groups may be carbohydrate residues, for example, monosaccharides, or polyethylene glycol derivatives. Examples of water solubilising constituents include  $C_1 - C_6$  alkyl sulphonates, such as  $-(CH_2)_3-SO_3^-$  and  $-(CH_2)_4-SO_3^-$ . However, one or more sulphonate or sulphonic acid groups attached directly to the aromatic ring structures of a dye of formula (II) or (III) are particularly preferred. Water solubility may be advantageous when labelling proteins.

In the embodiments according to the first aspect:

- i) Aryl is an aromatic substituent containing one or two fused aromatic rings containing 6 to 10 carbon atoms, for example phenyl or naphthyl, the aryl being optionally and independently substituted by one or more substituents, for example halogen, straight or branched chain alkyl groups containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;
- ii) Heteroaryl is a mono- or bicyclic 5 to 10 membered aromatic ring system containing at least one and no more than 3 heteroatoms which may be selected from N, O, and S and is optionally and independently substituted by one or more substituents, for example halogen, straight or branched chain alkyl groups containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;

- iii) Aralkyl is a C<sub>1</sub> – C<sub>6</sub> alkyl group substituted by an aryl or heteroaryl group;
- iv) Halogen and halo groups are selected from fluorine, chlorine, bromine and iodine.

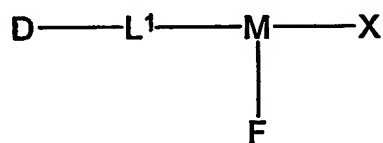
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By virtue of the target bonding group F, the compounds according to the present invention are useful for covalently labelling target biological materials in a site-specific manner for applications in biological detection systems. Suitable target materials include proteins, post-translationally modified proteins, peptides, antibodies, antigens, and protein-nucleic acids (PNAs). The reporter moiety may also be conjugated to species which can direct the path of the reporter within or aid entry to or exit from cells (live or dead); such as for example, long alkyl residues to allow permeation of lipophilic membranes, or intercalating species to localise a reporter in a nucleus or other cellular enclave containing double-stranded DNA.

10

In a second aspect, there is provided a method for labelling a protein of interest wherein said protein contains or is derivatised to contain an N-terminal cysteine, the method comprising:

- 20 i) adding to a liquid containing said protein a compound of formula (I):



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(I)

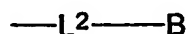
wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

30 M is a group adapted for attaching to F;

X is selected from hydrogen or the group:



wherein B is an affinity tag; and

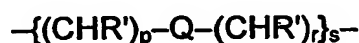
L<sup>1</sup> and L<sup>2</sup> each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl

5 groups, where R' is selected from hydrogen and C<sub>1</sub> – C<sub>4</sub> alkyl; and

ii) incubating said compound with said protein under conditions suitable for labelling said protein.

10 Suitably, there are 2 to 30 atoms in each of L<sup>1</sup> and L<sup>2</sup>, preferably, 6 to 20 atoms.

Preferably, L<sup>1</sup> and L<sup>2</sup> are independently selected from the group:



15

where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C<sub>1</sub> – C<sub>4</sub> alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

20 Particularly preferred Q is selected from: –CHR'–, –O– and –CO–NH–, where R' is hereinbefore defined.

Preferably, M is selected from:

25



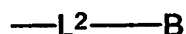
wherein R' is hereinbefore defined.

30 Covalent labelling using compounds of the present invention may be accomplished with a target having at least one carboxylic acid thioester group or 1,2-aminothiol group as hereinbefore defined. The target may be incubated with an amount of a compound of the present invention having at least one group F as hereinbefore defined that can covalently bind with the

complementary group of the target material. The target material and the compound of the present invention are incubated under conditions and for a period of time sufficient to permit the target material to covalently bond to the compound of the present invention. Thus, for example, the thioester group F  
5 may be reacted and form a covalent bond with any of the above target materials that contains, or has been derivatised to contain, a 1,2-amino thiol group. These methods and the products resulting from them, for example, reporter-labelled biomolecules are envisaged as further aspects of the invention.

10 Suitably, the protein of interest may be selected from the group consisting of antibody, antigen, protein, peptide, microbial materials, cells and cell membranes.

15 In one embodiment according to the second aspect, there is provided a method of separating and/or purifying the dye-labelled protein of interest by affinity chromatography. In this embodiment, compounds according to the invention include the group:



20 wherein B and  $\text{L}^2$  are hereinbefore defined. The method utilises the affinity of the affinity tag B covalently attached to the dye, for an immobilised ligand (or specific binding partner) attached to a support material. Affinity chromatography provides a quick and convenient method to enable the  
25 separation of labelled and unlabelled protein molecules under physiological conditions. Proteins labelled with an affinity tag can be selectively bound to an affinity column and any unreacted protein removed by washing the column. Suitable specific binding moieties include avidin or streptavidin (for a biotin tag); immobilised metal ions, for example Cu(II), Ni(II), Fe(II) and Fe(III) (for  
30 His-tag or iminodiacetic acid). Methods for affinity purification of proteins will be well known to the skilled person, see for example Ostrove, S., Methods in Enzymology, (1990), Vol 182, page 357.

In a typical labelling procedure, a target peptide or protein containing an N-terminal cysteine residue is agitated with an excess of an acridone or quinacridone dye thioester derivative, for example, 9-oxo-10-{6-oxo-6-[2-sulfoethyl]thio]hexyl}-9,10-dihydroacridine-2-sulphonic acid (Ace-MESNA) in  
5 buffer (typically 200 mM NaCl, 200 mM sodium phosphate) at ~pH 7.3 – 7.4 containing ~1.5% MESNA. The concentration of the target polypeptide in the labelling reaction is generally between 100  $\mu$ M to 10 mM, whilst the Ace-MESNA is generally present in excess, for example 1.5 to 3-fold molar excess. When the target polypeptide concentration is relatively low, the  
10 concentration of Ace-MESNA is usually maintained at or above 1 mM. Generally, for labelling small peptides a solution of Ace-MESNA and MESNA cofactor is directly added to the lyophilised target.

Typically, for site specific labelling of proteins and large polypeptides  
15 using the reagents of the present invention, the target is first transferred into an appropriate buffer, which is known not to effect the labelling reaction. An equal volume of a solution of Ace-MESNA and MESNA thiol co-factor in ligation buffer is then added to the protein to give the desired final concentration of the reactants. The reaction mixture is agitated overnight at  
20 room temperature. The reaction time may be lowered to less than one hour for high reactant concentrations and, if the stability of the target polypeptide is an issue, the labelling reaction can be performed efficiently at 4°C. On completion of the labelling reaction, dithiothreitol (DTT) is added to a final concentration of ~50 – 250 mM and the desired material isolated by a  
25 chromatographic procedure.

Various different denaturants, organic solvents and detergents may be added to the reaction buffer when performing native chemical ligation and expressed protein ligation reactions, to aid the ligation of the peptide  
30 fragments and/or stabilise the reactants or products. Such reagents may be utilised in the labelling reaction to increase product yield if necessary. Examples include, but are not limited to guanidinium chloride, urea,

dimethylformamide, dimethylsulfoxide, acetonitrile, triton X-100, octyl glucoside, 1,6-hexanediol and glycerol.

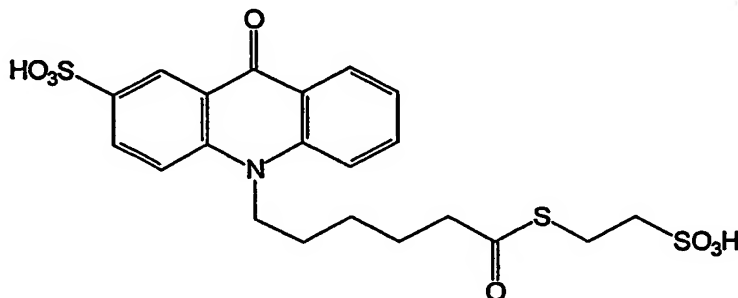
The ligation reaction using a derivatised acridone or quinacridone dye according to the present invention may be optimally performed at between pH 7.0 and pH 8.0 and at temperatures varying between 4°C and 37°C. It is envisaged that such a range of conditions are compatible to the site-specific labelling reaction described herein.

The advantage of the present method is that it enables the introduction of an extrinsic label into a proteinaceous substrate in a regioselective and specific manner, thus minimising any detrimental effects that labelling may have on the biological function of the protein. The importance of controlling stoichiometry of labelling is important where dye overload may interfere with biological activity. In addition, if this controlled labelling stoichiometry is directed towards a single terminal site, rather than towards an internal site, this may have the benefit of further maintaining the biological viability of the labelled species.

The invention is further illustrated by reference to the following examples.

### Experimental

1. 9-Oxo-10-{6-oxo-6-[2-sulfoethyl]thio}hexyl}-9,10-dihydroacridine-2-sulphonic acid (Ace-MESNA)



To a stirred solution of 9-oxo-10-[6-carboxyhexyl]-9,10-dihydroacridine-2-sulphonic acid (38.9mg, 0.1mmol) and 2-mercaptoethylsulphonic acid, sodium salt (MESNA) (25.2mg, 0.153mmol) in anhydrous dimethylformamide (3ml) at ambient temperature was added a solution of dimethylaminopyridine (13.4mg, 0.11mmol) in anhydrous dimethylformamide (1.25ml) followed by a solution of 1-hydroxybenzotriazole (17.9mg, 0.132mmol) in anhydrous dimethylformamide (0.5ml). To this mixture was added as a solid, dried 4A molecular sieves (~1g, <5micron, activated, powder). The mixture was allowed to stir under a dry nitrogen atmosphere for 30 minutes, and this was followed by addition of N,N'-diisopropylcarbodiimide (126mg, 155ul, 1mmol). The mixture was stirred under a dry nitrogen atmosphere for 15 hours. Thin layer chromatography analysis (reverse phase C18 plates, eluent water/acetonitrile (70:30, containing 0.1% TFA) indicated a major UV visible component (rf 0.43) with no trace of starting material (reference rf 0.34). Work up was by filtration through a glass sinter funnel (porosity 3), washing with dimethylformamide (10ml), pouring onto cold diethylether (30ml), and centrifuging (5-10° C, 100rpm, 10 minutes). After trituration with ethylacetate (2x 20ml) and dissolving the dispersed solid into water (25ml), HPLC purification was carried out (Phenomenex Jupiter C18 column, 0-60% gradient elution of water/acetonitrile (containing 0.1% TFA) over 40 minutes. The product was obtained as a yellow solid (28.8mg, 56%), m/z (Maldi) 514, NMR  $\delta_H$  (300MHz, D<sub>2</sub>O) 1.45 (2H, m), 1.72 (4H, m), 2.54 (2H, t, CH<sub>2</sub>-CO-S), 3.19 (4H, m, SCH<sub>2</sub>CH<sub>2</sub>), 4.24 (2H, t, NCH<sub>2</sub>), 7.23-8.15 (6H, m, aromatic H) and 8.53 (1H, s, 1-CH).

## 2. Synthesis of Ace-Cys-Gly-Leu-Asp-Lys-Arg -Gly-Cys-Gly-NH<sub>2</sub>

### 2.1 H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Arg(Pmc)-Lys(Boc)Gly-Cys(Trt)Gly-rink amide resin

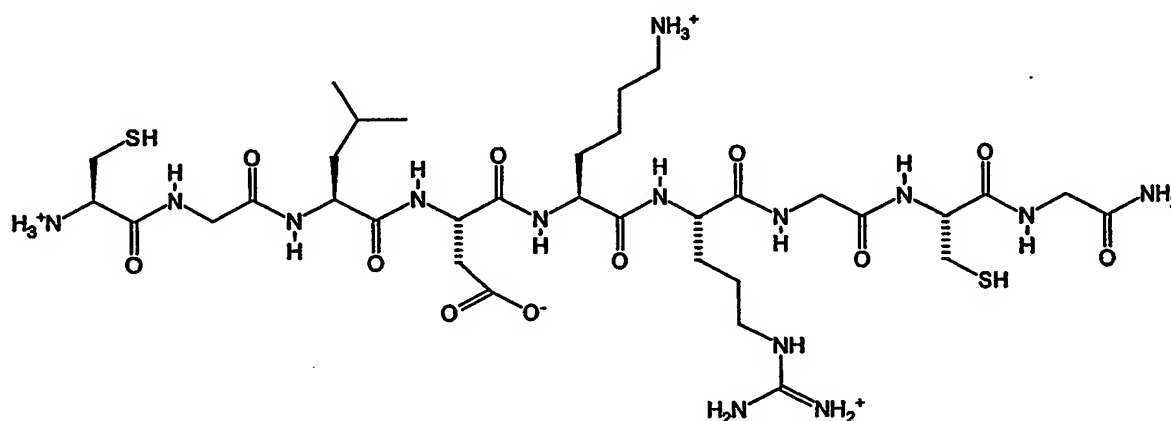
H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Arg(Pmc)-Lys(Boc)Gly-Cys(Trt)Gly-rink amide resin was synthesised using a commercially available Applied Biosystems Model 433A automated peptide synthesiser using FastMoc™

chemistry, following the instrument manufacturer's recommended procedures throughout. The peptide was synthesised on a 0.25 millimolar scale employing O-(benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activating agent.

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## 2.2 Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>

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15

H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Lys(Boc)-Arg(Pmc)-Gly-Cys(Trt)-Gly-rink amide resin (100mg, theoretical loading 0.36mmol/g) was deprotected and cleaved from solid phase in 95% trifluoroacetic acid (TFA) / 2.5%tri-isopropylsilane (TIS) / 2.5% water (3 mls) at room temperature for 2 hours. The crude product was precipitated into a 10 fold excess of cold diethyl ether, centrifuged at 2500 rpm for 5 minutes and the ether decanted off. The crude peptide was washed twice more with ether and was purified by reverse phase-high performance liquid chromatography (RP-HPLC) [Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient : 0-73%B over 30 mins @1ml/min, detection at 214nm ]. The product was isolated and lyophilised to afford a colourless fluffy solid (21mg by weight, 60%). Mono-isotopic mass (as carboxylate): 906.09. Found mass (LC-MS): MH+ @ 907.3; M+Na @ 929.6; > 95% pure as judged by RP-HPLC @ 214nm (Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, 5-50% B over 25mins @ 1ml /min, UV detection at 650nm).

30

### 2.3 Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>

To solid H-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub> (3.2mg by weight, 0.0035mmol) was added a solution of Ace-MESNA (2.5mg, 0.0049mmol, 1.4 equivalents) in 200mM phosphate/200mM NaCl buffer pH 7.2 containing 1.5% 2-mercaptoethanesulphonic acid, sodium salt (500μl). The reaction mixture was stirred for 30 minutes at room temperature in darkness. During incubation, a precipitate formed, which re-dissolved on manual agitation.

A 50μl aliquot of the reaction mix was removed and to this was added 200mM phosphate buffer/200mM NaCl pH 7.2 containing 250mM (final) dithiothreitol (DTT) (100μl). The crude reaction mixture was then analysed by RP-HPLC [250 x 4.6mm Phenomenex Jupiter C4 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 35 mins at 1ml/min, detection at 220nm and 400nm]. HPLC indicated over 90% consumption of dye (rt. ~14.7min), with major new 400nm-visible peaks at rt. 18.91, 21.60 and 23.42 mins. The remainder of the reaction mix was then worked up by addition of 200mM phosphate buffer/200mM NaCl pH 7.2 containing 250mM (final) dithiothreitol (DTT) (200μl). The crude reaction mixture was then subjected to 3 semi-preparative RP-HPLC runs [250 x 10mm Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 40mins at 5ml/min, detection at 220nm and 400nm]. The second of four 400nm-visible peaks (rt. ~26.5mins) was identified as the desired product (MALDI-MS, mono-isotopic mass C<sub>53</sub>H<sub>78</sub>N<sub>14</sub>O<sub>17</sub>S<sub>3</sub> requires 1277, found MH<sup>+</sup> 1278). The product from the third HPLC run was isolated and lyophilised as a pale yellow solid (1.4 mg by weight; ~54.5% yield; 97% pure as judged by RP-HPLC at 400nm wavelength. Additional identification was carried out: (LC-MS ES<sup>+</sup> single component gives MH<sup>+</sup> @ 1278).

## 2.4 Characterisation of Labelled Peptide

### 2.4.1 Enzyme Digestion of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>

5 To a solution of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>  
(~750µg from the first two preparative HPLC runs from Experimental Section  
4.3) in 50mM TRIS buffer pH 7.9 (500µl) containing 0.005% Tween was  
added Endo Asp-N (2µg) in 50mM TRIS buffer pH 7.9 (100µl). The reaction  
mixture was stirred overnight at room temperature in the dark under an  
10 atmosphere of nitrogen. The reaction mixture was treated with 50mM TRIS  
buffer pH 7.9 (200µl) containing 250mM (final) dithiothreitol for 30 minutes.  
The crude reaction mixture was then analysed by RP-HPLC [250 x 4.6mm  
Phenomenex Jupiter C4 column, eluent A: 0.1%TFA/water, eluent B:  
0.1%TFA/acetonitrile, gradient; 0-50%B over 35 mins at 1ml/min, detection at  
15 220nm and 400nm]. HPLC indicated virtual complete enzyme digestion, with  
a major new 400nm-visible peak at rt. 24.71min and trace of starting substrate  
at rt. 23.09mins. The crude reaction mixture was then subjected to semi-  
preparative RP-HPLC [250 x 10mm Phenomenex Jupiter C18 column, eluent  
A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over  
20 35mins at 5ml/min, detection at 220nm and 400nm]. The main 400nm-visible  
peak (rt. ~31.18mins) was identified as the desired product (97% by peak  
area) (MALDI-MS, Ace-Cys-Gly-Leu-OH, mono-isotopic mass C<sub>30</sub>H<sub>37</sub>N<sub>4</sub>O<sub>9</sub>S<sub>2</sub>  
requires 661.78. Found M+H @ 663). In addition, the crude reaction mixture  
was analysed (LC- MS ES+, diode array detection). Required cleavage  
25 product A (Ace-Cys-Gly-Leu) requires 662.79, found MH+ 663. Required  
cleavage product B (Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>) requires 634, found MH+  
634. There was no evidence for compound peaks corresponding to non-  
specific internal Cys residue labelling. The non-cleaved starting material  
(Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>) was also observed as  
30 MH<sub>2</sub><sup>2+</sup> 639.6 (MH+ @1278.2), indicating an endoAsp N enzyme cleavage  
efficiency of 91.8%.

#### 2.4.2 MS Sequence Analysis of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH<sub>2</sub>

Sequence analysis (MS/MS ion sequence  $\beta$  and  $\gamma$  directions) shows  
5 consistency with structure with the terminal Ace-Cys residue observed @ M+  
475.06. No evidence of internal Cys labelling was observed in the sequence.

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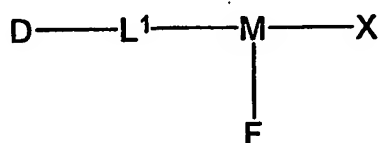
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Claims

1. A compound selected from an acridone and a quinacridone dye containing at least one target bonding group selected from a carboxylic acid thioester group or a group suitable for covalent reaction with a thioester, wherein said compound optionally includes an affinity tag covalently bound thereto.

2. A compound according to claim 1 having the formula (I):

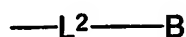


(I)

wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;  
F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;  
M is a group adapted for attaching to F;

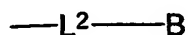
X is selected from hydrogen or the group:



wherein B is an affinity tag; and

L<sup>1</sup> and L<sup>2</sup> each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C<sub>1</sub> – C<sub>4</sub> alkyl.

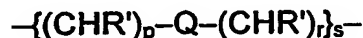
3. A compound according to claim 2 wherein X is the group:



wherein B and L<sup>2</sup> are hereinbefore defined.

4. A compound according to claim 2 or claim 3 wherein each of L<sup>1</sup> and L<sup>2</sup> contains from 2 to 30 atoms.

5. A compound according to claim 2 or claim 3 wherein L<sup>1</sup> and L<sup>2</sup> are  
5 independently selected from the group:



where Q is selected from: -CHR'-, -NR'-, -O-, -CH=CH-, -Ar- and  
10 -CO-NH-; R' is hydrogen or C<sub>1</sub> - C<sub>4</sub> alkyl, p is 0 - 5, r is 1 - 5 and s is 1 or 2.

6. A compound according to claim 5 wherein Q is selected from -CHR'-, -O- and -CO-NH-, where R' is hereinbefore defined.

15 7. A compound according to any of claims 1 to 6 wherein said affinity tag is selected from biotin and desthiobiotin.

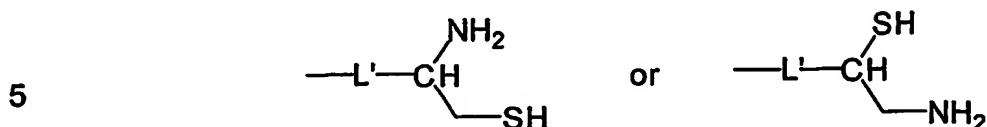
8. A compound according to any of claims 1 to 6 wherein said affinity tag is selected from his-tag, iminodiacetic acid and nitrilotriacetic acid.  
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9. A compound according to any of claims 1 to 8 wherein the target bonding group F is a carboxylic acid thioester of formula:



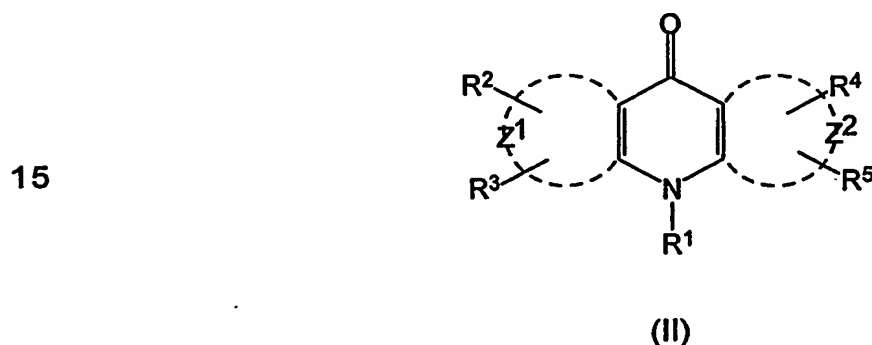
wherein L' is a bond or is a group containing from 1 - 30 linked atoms selected from carbon atoms and optionally one or more groups selected from -NH-, -O- and -CO-NH-; and R'' is C<sub>1</sub> - C<sub>4</sub> alkyl, C<sub>6</sub> - C<sub>10</sub> aryl, or C<sub>7</sub> - C<sub>15</sub> aralkyl, which may be optionally substituted with sulphonate; or is the group  
30 -(CH<sub>2</sub>)<sub>2</sub>-CONH<sub>2</sub>.

10. A compound according to any of claims 1 to 8 wherein the target bonding group F is a 1,2-aminothiol group of formula:



wherein L' is hereinbefore defined.

11. A compound according to any of claims 1 to 10 wherein the compound is an acridone dye having the formula (II):



wherein:

20 groups R<sup>2</sup> and R<sup>3</sup> are attached to the Z<sup>1</sup> ring structure and groups R<sup>4</sup> and R<sup>5</sup> are attached to the Z<sup>2</sup> ring structure;  
 Z<sup>1</sup> and Z<sup>2</sup> independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms  
 25 selected from oxygen, nitrogen and sulphur;  
 at least one of groups R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> is a group W having the formula:



where F, M, X and L<sup>1</sup> are hereinbefore defined;

when any of said groups  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  is not said group W, said remaining groups  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  are independently selected from hydrogen, halogen, amide, cyano, mono- or di- $C_1 - C_4$  alkyl-substituted

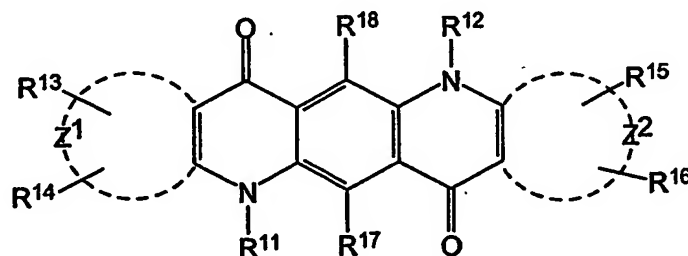
amino, carbonyl, carboxyl,  $C_1 - C_6$  alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl,  $C_1 - C_{20}$  alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group  $-(CH_2)_n-Y$  and,

when group  $R^1$  is not said group W, it is selected from hydrogen,  $C_1 - C_{20}$  alkyl, aralkyl and the group  $-(CH_2)_n-Y$ ; and

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

provided that at least one of groups  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  is a water solubilising group.

12. A compound according to any of claims 1 to 10 wherein the compound is a quinacridone dye having the formula (III):



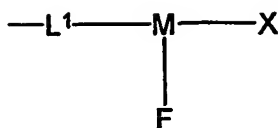
(III)

wherein:

groups  $R^{13}$  and  $R^{14}$  are attached to the  $Z^1$  ring structure and groups  $R^{15}$  and  $R^{16}$  are attached to the  $Z^2$  ring structure;

$Z^1$  and  $Z^2$  independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$  and  $R^{18}$  is a group T having the formula:



- 5 where F, M, X and L<sup>1</sup> are hereinbefore defined;  
 when any of said groups R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>17</sup> and R<sup>18</sup> is not said group T,  
 said remaining groups R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>17</sup> and R<sup>18</sup> are independently  
 selected from hydrogen, halogen, amide, cyano, mono- or di-C<sub>1</sub> – C<sub>4</sub> alkyl-  
 substituted amino, carbonyl, carboxyl, C<sub>1</sub> – C<sub>6</sub> alkoxy, acrylate, vinyl, styryl,  
 10 aryl, heteroaryl, C<sub>1</sub> – C<sub>20</sub> alkyl, aralkyl, sulphonate, sulphonic acid, quaternary  
 ammonium and the group –(CH<sub>2</sub>)<sub>n</sub>–Y; and,  
 when either of groups R<sup>11</sup> and R<sup>12</sup> is not said group T, it is selected from  
 hydrogen, C<sub>1</sub> – C<sub>20</sub> alkyl, aralkyl and the group –(CH<sub>2</sub>)<sub>n</sub>–Y;  
 Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary  
 15 ammonium and carboxyl; and n is an integer from 1 to 6;  
 provided that at least one of groups R<sup>11</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>17</sup> and R<sup>18</sup> is  
 a water solubilising group.

13. A compound according to claim 11 or claim 12 wherein Z<sup>1</sup> and Z<sup>2</sup> are  
 20 selected independently from the group consisting of phenyl, pyridinyl,  
 naphthyl, quinolinyl and indolyl moieties.

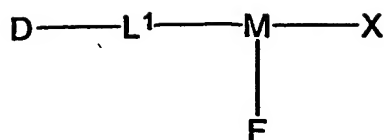
14. A compound according to claim 11 or claim 12 wherein Z<sup>1</sup> and Z<sup>2</sup> are  
 selected from phenyl and naphthyl moieties.

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15. A method for labelling a protein of interest wherein said protein  
 contains or is derivatised to contain an N-terminal cysteine, the method  
 comprising:

- i) adding to a liquid containing said protein a compound of formula (I):

30



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(I)

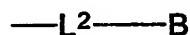
wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

10 M is a group adapted for attaching to F;

X is selected from hydrogen or the group:



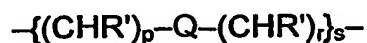
where B is an affinity tag; and

15 L<sup>1</sup> and L<sup>2</sup> each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C<sub>1</sub> – C<sub>4</sub> alkyl; and

20 ii) incubating said compound with said protein under conditions suitable for labelling said protein.

16. A compound according to claim 15 wherein each of L<sup>1</sup> and L<sup>2</sup> contains from 2 to 30 atoms.

25 17. A method according to claim 15 wherein L<sup>1</sup> and L<sup>2</sup> are independently selected from the group:

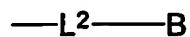


30 where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C<sub>1</sub> – C<sub>4</sub> alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

18. A method according to claim 17 wherein Q is selected from  $-\text{CHR}'-$ ,  $-\text{O}-$  and  $-\text{CO}-\text{NH}-$ , where R' is hereinbefore defined.

19. A method according to any of claims 15 to 18 wherein X is the group:

5



wherein B and  $\text{L}^2$  are hereinbefore defined, said method further comprising separating and/or purifying the dye-labelled protein of interest by affinity chromatography.

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20. A method according to any of claims 15 to 19 wherein said protein of interest is selected from antibody, antigen, protein, peptide, microbial materials, cells and cell membranes.

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# INTERNATIONAL SEARCH REPORT

International application No  
PCT/L 03/03190

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 G01N33/58 C09B15/00 C07K7/06		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 G01N C09B C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 835 139 A (PFISTER J ET AL) 10 September 1974 (1974-09-10) example 13	1
Y	FALLER T ET AL: "A novel acridone derivative for the fluorescence tagging and mass spectrometric sequencing of peptides" CHEMICAL COMMUNICATIONS, ROYAL SOCIETY OF CHEMISTRY, GB, vol. 16, 1997, pages 1529-1530, XP002251180 ISSN: 1359-7345 page 1529, left-hand column, the last three paragraphs --- -/--	1-20
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family		
Date of the actual completion of the international search 27 October 2003		Date of mailing of the international search report 10/11/2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Fausti, S

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/JP 03/03190

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHULER BENJAMIN ET AL: "Specific labeling of polypeptides at amino-terminal cysteine residues using Cy5-benzyl thioester." BIOCONJUGATE CHEMISTRY. UNITED STATES 2002 SEP-OCT, vol. 13, no. 5, 18 July 2002 (2002-07-18), pages 1039-1043, XP002259205 ISSN: 1043-1802 cited in the application abstract; page 1039, right-hand column, first paragraph; schemes 1 and 2 -----	1-20
P,Y	WO 02 099424 A (AMERSHAM BIOSCIENCES UK LTD ;WEST RICHARD MARTIN (GB); SMITH JOHN) 12 December 2002 (2002-12-12) claims 1,7,11,13,27-29 -----	1-11, 13-20
P,Y	WO 02 099432 A (AMERSHAM BIOSCIENCES UK LTD ;SMITH JOHN ANTHONY (GB)) 12 December 2002 (2002-12-12) claims 1,6,9,11,27-29 -----	1-10, 12-20
A	KLEIN G ET AL: "A fluorescent metal sensor based macrocyclic chelation" CHEMICAL COMMUNICATIONS, CHEMICAL SOCIETY, LONDON, GB, vol. 7, no. 6, 21 March 2001 (2001-03-21), pages 561-562, XP002227157 ISSN: 0009-241X abstract; scheme 1 -----	12
A	EP 0 857 721 A (EISAI CO LTD) 12 August 1998 (1998-08-12) production example 9; examples 1,2; table 5 -----	1

# INTERNATIONAL SEARCH REPORT

Inter al application No.  
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## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1,7,11-14 (all partially)  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1,7,11-14 (all partially)

Present claim 1 relates to an acridone or quinacridone dye containing a target bonding group, which is partially defined by reference to a desirable characteristic or property, namely its reaction with a thioester.

Claims 1, 7 and 11-14 cover all compounds, whose target bonding group has this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the acridone or quinacridone dyes, whose target bonding group is a carboxylic acid thioester group (as clearly defined in independent claim 1) or is a 1,2-aminothiol group (see for example claims 2 and 10).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 03/03190

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 3835139	A	10-09-1974	GB 1417201 A	10-12-1975
			US 3886162 A	27-05-1975
WO 02099424	A	12-12-2002	WO 02099424 A2	12-12-2002
WO 02099432	A	12-12-2002	WO 02099432 A2	12-12-2002
EP 0857721	A	12-08-1998	AU 7145396 A	28-04-1997
			EP 0857721 A1	12-08-1998
			CA 2233643 A1	10-04-1997
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